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MUSCLE ATTACHMENTS IN HORSESHOE CRAB WALKING LEGS

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The attachment of muscle to skeletal elements in arthropods has been the subject of electron microscopical studies in insects (Auber, 1963; Shafiq, 1963; Lai-Fook, 1967; Toselli and Pepe, 1968a, 1968b; Caveney, 1969), arachnids (Smith, Jälfors and Russell, 1969; Kuo, McCully and Haggis, 1971; Mazurkiewicz and Bertke, 1972) and crustaceans (Bouligand, 1962; Talbot, Clark and Lawrence, 1972; Koulish, 1973). The results of these studies reveal that the attachment region is similar in structure throughout the arthropods that have been examined. The attachment may be made directly to the exoskeleton, but in most cases is achieved through a highly specialized epidermal cell, usually called a tendinal cell, interposed between a muscle fiber and the cuticle. The most conspicuous feature of the tendinal cells is their tremendous concentration of oriented microtubules which extend the length of the cell and join desmosomal and hemidesmosomal junctions formed between muscle and tendinal cell and tendinal cell and apodeme respectively.

At the present time, no studies of muscle attachments utilizing electron microscopical techniques have been reported for a fourth major class of arthropods, the Merostomata. Such a study of members of this group which includes the oldest extant arthropods, the horseshoe crabs, would be particularly interesting in view of their long evolutionary history. This report is concerned with the ultrastructural details of the attachment of propodite flexor muscle fibers to the exoskeleton in *Limulus polyphemus* (L.). The results show that for the most part, these muscle attachments in horseshoe crabs follow the pattern described for other members of the Arthropoda.

Materials and Methods

Adult female horseshoe crabs, *Limulus polyphemus* (L.), having a carapace width of about 21 cm, were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were prepared for electron microscopy in March during their intermolt stage. The second, third and fourth pairs of walking legs were used. A leg was removed at the coxopodite, secured to a glass petri dish with dental wax and submerged in artificial sea water (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio) at room temperature. The exoskeleton overlying the propodite flexor muscle heads was removed to expose the regions of muscle attachment at both the origin and insertion ends. A detailed description of the gross morphology and ultrastructure of the propodite flexor muscle is given elsewhere (Fourtner and Sherman, 1972).

After the muscle was exposed, it was fixed *in situ* for two hours in a solution containing 4% glutaraldehyde, 0.1 M sodium cacodylate buffer (ph 7.3), 2% sodium chloride and 0.2% calcium chloride. It then was washed in a solution

containing only the above salts and fixed for one hour in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate (pH 7.3). The muscle was again placed in the salts solution and a number of pieces of muscle tissue were removed from different regions of both the origin and insertion ends of the muscle. This was accomplished with a piece of razor blade held by a blade breaker and holder (Model IR-471, Irex Surgical Instruments, Toronto, Canada). The tissues were prestained for two hours in a saturated solution of uranyl acetate in 50% ethanol, dehydrated in a graded ethanol series, cleared in propylene oxide and embedded in an epon-araldite mixture. Thin sections were stained with ethanolic uranyl acetate and lead citrate (Reynolds, 1963) and examined with a transmission electron microscope.

Results

Two modes of muscle fiber attachment occur in the propodite flexor muscle of *L. polyphemus*. At the insertion end, the attachment involves three elements: muscle fibers, tendinal cells and apodemes. At the origin, muscle fibers are attached directly to the exoskeleton by collagen fibrils.

Characteristics of epidermal cells

Sections through apodemes reveal basically two types of epidermal cell. One type, presumably fibroblasts, is embedded in the matrix of the apodeme and probably is responsible for secreting the cuticle (Fig. 1). This cell type is irregular in shape and its nucleus occupies most of the cell volume. The fibroblasts are about 8 to 10 μ at their greatest width. The cytoplasm typically shows a relative scarcity of subcellular structures. In contrast to the second type of epidermal cell, these cells do not form specialized junctions with apodemes or muscle fibers and they do not contain microtubules.

The second type of epidermal cell corresponds to the tendinal cells described previously for other arthropods. These cells are more abundant, contain tremendous numbers of oriented microtubules and form specialized junctions with muscle fibers and apodemes (Fig. 2). They are irregular in shape and interdigitate profusely with muscle and apodemal elements. Besides microtubules and the nucleus, the tendinal cells primarily contain moderate amounts of mitochondria, free ribosomes and single-stranded rough endoplasmic reticulum. Small clear vesicles also are present in most cells. Several tendinal cells often occur in close association with one another (Fig. 3).

The microtubules in the tendinal cells are about 210 Å in diameter. They are not arranged into a particular pattern in the cytoplasm, but usually are within 50 to 150 Å from one another. The microtubules may reach a concentration as high as $910/\mu^2$ in certain cells. They extend the entire length of the tendinal cell which in some cases involves a distance of 20 μ.

In cases where two or more epidermal cells occur together, they are linked by both septate and gap junctions (Figs. 4 and 5). The septate junctions consist of an intercellular gap of about 100 Å and transverse septa spaced about 100 Å apart (Fig. 5). These junctions run uninterrupted for distances of several microns along the borders of neighboring cells. The gap junctions are characterized by a separation of about 30 Å between the apposing membranes at the junctional re-

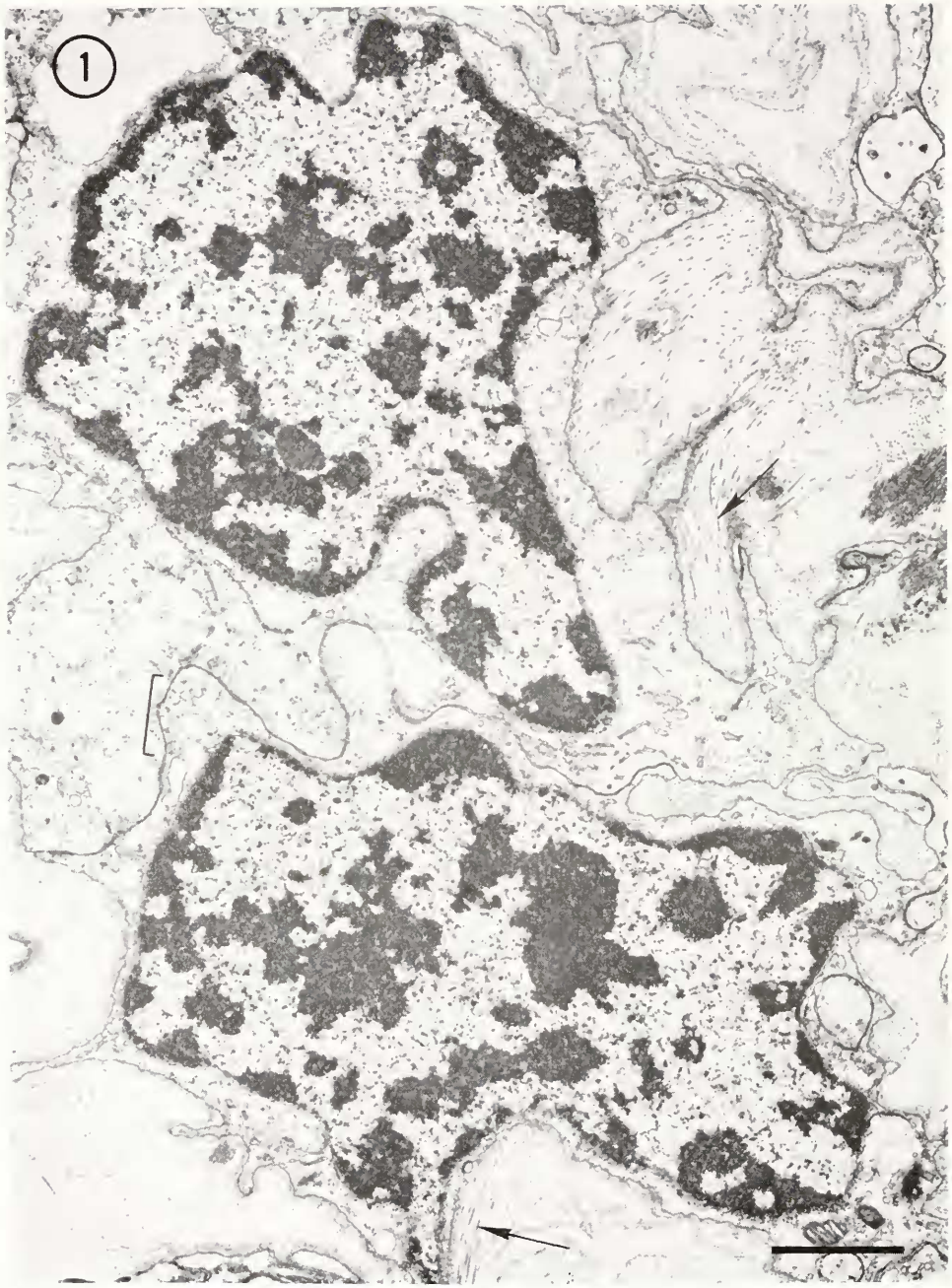

FIGURE 1. Two epidermal cells, presumed to be fibroblasts, are embedded in cuticle. This type of cell lacks appreciable subcellular detail, and the nucleus occupies the bulk of the cell volume. Note the collagen fibrils (arrow) in the cuticle. The area designated by the bracket contains a gap junction which is shown at higher magnification in Figure 4. Calibration line represents 1.3 μ.

FIGURE 2. Parts of a number of tendinal cells attached to apodeme (asterisks) through hemidesmosomal junctions. The nucleus of one of the tendinal cells is shown as is the close apposition of the membranes of adjacent cells (arrow). Note the tremendous number of microtubules (Mt) oriented longitudinally in the elongated tendinal cells; dense material lines, in a discontinuous manner, the inner surface of the tendinal cell membrane at the regions of attachment. Collagen fibrils are present in the surface cuticle (Cu) but not in the apodemal extensions (asterisks) which have an amorphous appearance. Calibration line represents 1.0 μ.

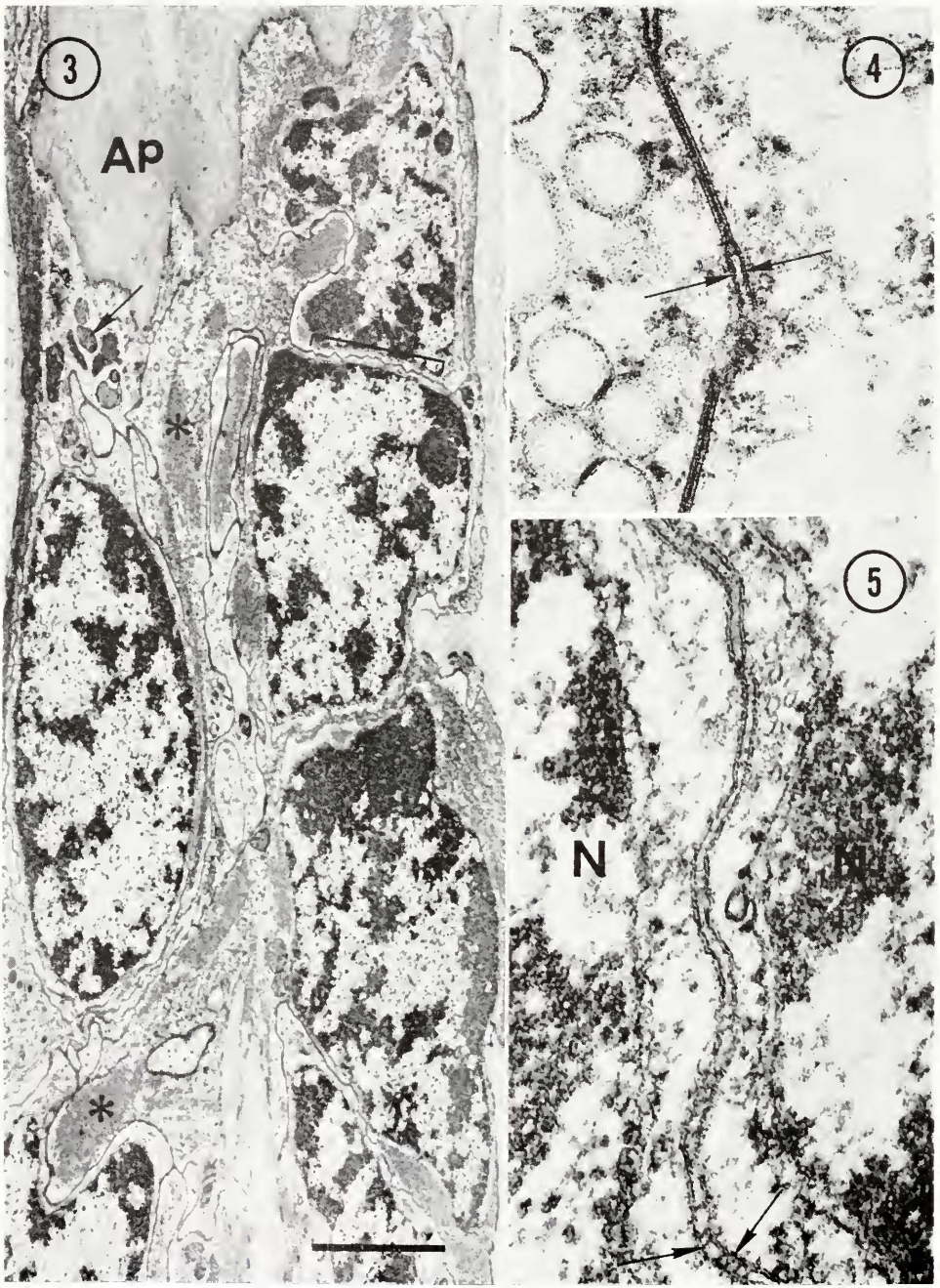

FIGURE 3. Parts of at least six different tendinal cells are shown in close association with one another near an apodeme (Ap). Nuclei, small mitochondria (arrow), very small vesicles and groups of microtubules (asterisks) occur in the cytoplasm. Note the close apposition of the

gions (Fig. 4). An analysis of the three-dimensional structure of these intercellular junctions was not undertaken.

Muscle-tendinal cell junction

The junction between muscle fibers and tendinal cells possesses the structural features of a desmosome and marks the extensive course of the cellular interdigitations (Figs. 6 and 7). Electron-dense material occurs along the inner surface of each apposing membrane at the junction. Thin myofilaments of muscle fibers and microtubles of tendinal cells extend into their respective layers of dense material (Fig. 6). The angle between the thin myofilaments or microtubules and the dense material varies between 15 and 90°. No thick myofilaments are present at the attachment site. Moderately-dense, amorphous basal lamina-like material occupies the space between the cell membranes at the desmosome.

The desmosomes are not continuous across a given muscle fiber. Generally, they are oriented transversely to the longitudinal axis of the muscle fiber, but their course is quite tortuous. The extracellular gap at the desmosome varies in width from about 400 to 1,000 Å. The layer of dense material along the inner surface of the membranes at the desmosome is absent from the neighboring non-junctional areas. Here, membrane invaginations of muscle fibers form tubular elements of the transverse tubular system (Fig. 6).

Tendinal cell-apodeme junction

The junction between the apical end of the tendinal cells and the apodeme is characterized by a considerable degree of invagination of the tendinal cells by "tongue-like" extensions of the apodeme (Figs. 2 and 7). The actual attachment occurs through hemidesmosomes. The microtubules in the tendinal cells connect to the electron-dense material which lines in a discontinuous fashion the inner surface of the tendinal cell membrane at the hemidesmosome. The apodemal indentations into the tendinal cells consist of moderately dense, amorphous material (Figs. 2 and 7). In contrast to the surface cuticle, the apodemal indentations do not contain collagen fibrils.

Direct myo-cuticular attachments

Tendinal cells were not observed at muscle fiber origins. Instead, muscle fibers appear to be connected directly to the exoskeleton by collagen fibrils (Fig. 8). The junction between the collagen fibrils and muscle fibers occurs at the level of

membranes of adjacent cells. The area designated by the bracket contains a septate junction and is shown at higher magnification in Figure 5. Calibration line represents 1.7 μ.

FIGURE 4. The area designated by the bracket in Figure 1 is shown at higher magnification to show the gap junctions that occur between adjacent epidermal cells. The arrows denote apposing cell membranes. Calibration line in Figure 3 represents 0.17 μ in Figure 4.

FIGURE 5. The area designated by the bracket in Figure 3 is shown at higher magnification to show the transverse septa that occur between certain areas of the membranes (arrows) of adjacent epidermal cells. The nuclei (N) of the two epidermal cells are evident. Unfortunately, no well-preserved septate junctions were located in the present study. Calibration line in Figure 3 represents 0.16 μ in Figure 5.

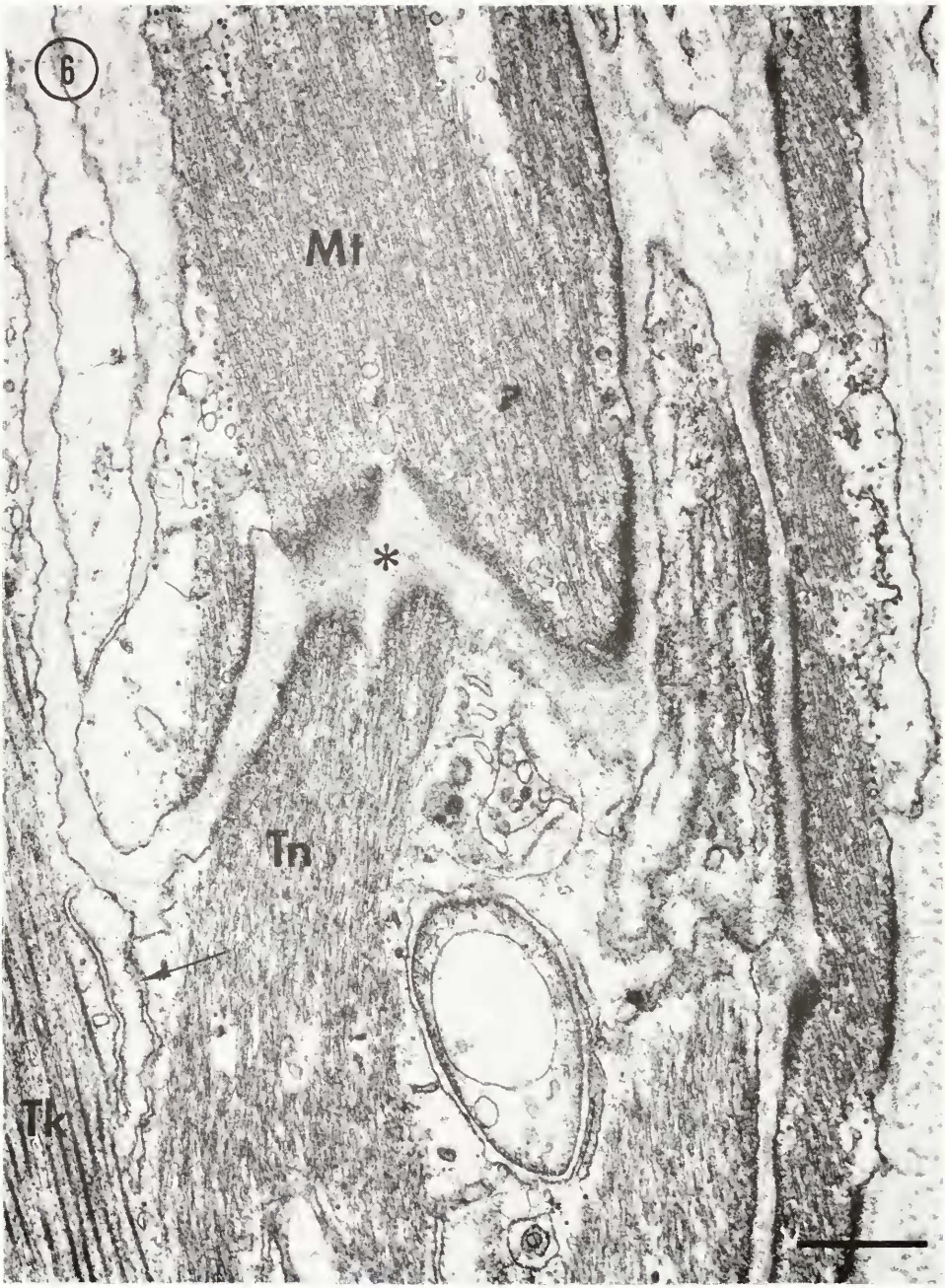

FIGURE 6. Muscle-tendinal cell desmosomal junction (asterisk) is shown. Microtubules (Mt) of the tendinal cell and thin myofilaments (Tn) of the muscle fiber extend into dense material lining the inner surface of the membranes at the junction. Thick myofilaments (Tk) are not directly involved in the attachment. Note the wide gap between the membranes at the junction. Plasma membrane invaginations (arrow) of the non-junctional region of the muscle fiber form part of the transverse tubular system. Calibration line represents 0.4 μ.

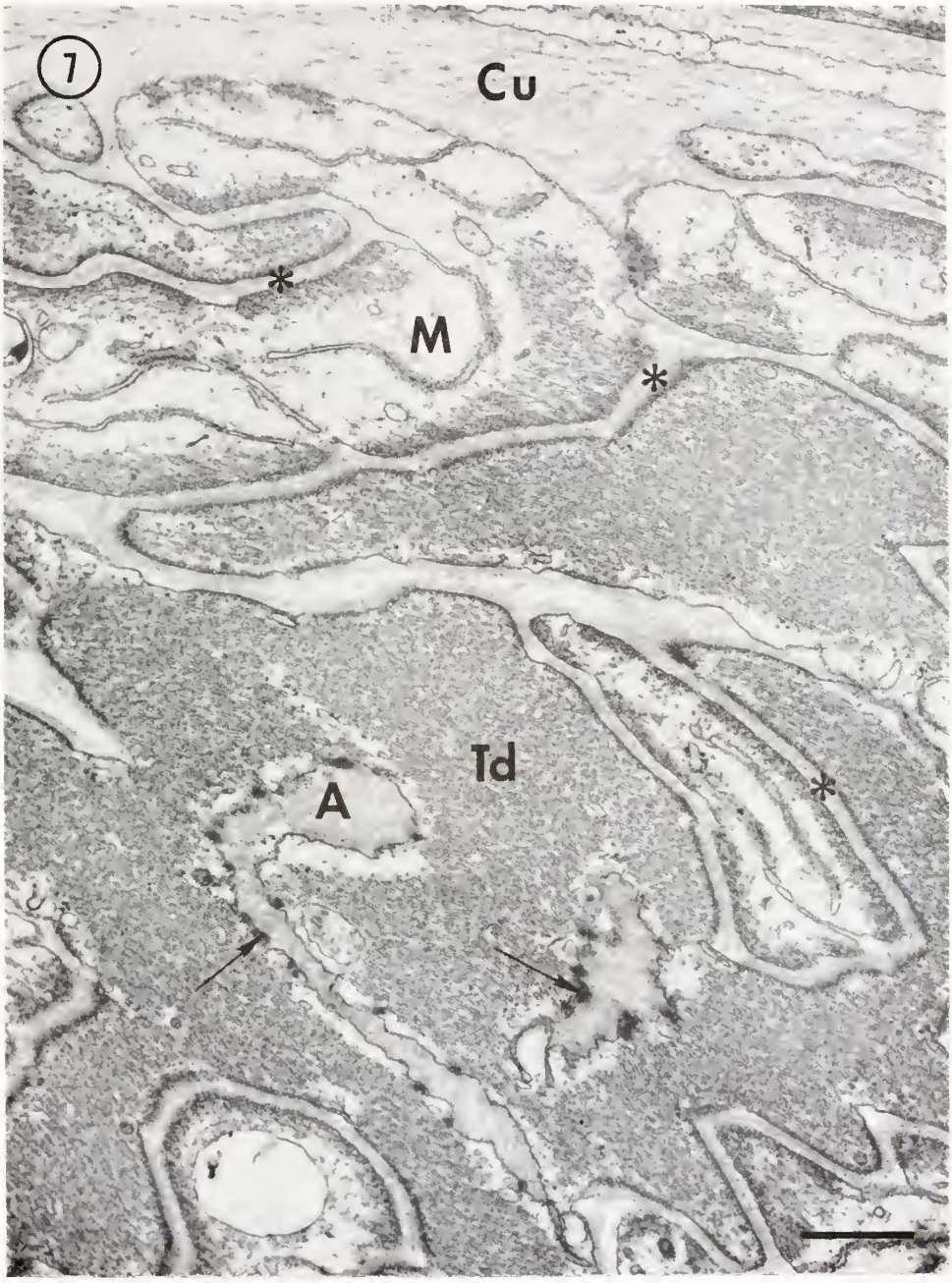

FIGURE 7. Both muscle-tendinal cell and tendinal cell-apodeme attachment sites are shown. The desmosomal-like muscle-tendinal junction (asterisks) shows a very extensive degree of interdigitation between muscle (M) and tendinal-cell (Td). The tendinal cell-apodeme (A) junction is hemidesmosomal (arrows) in nature. Here, dense material lines only the inner surface of the tendinal cell membrane. The attachment is formed along invaginations of the tendinal cell by "tongue-like" extensions of apodeme; Cu, cuticle. Calibration line represents 0.5 μ.

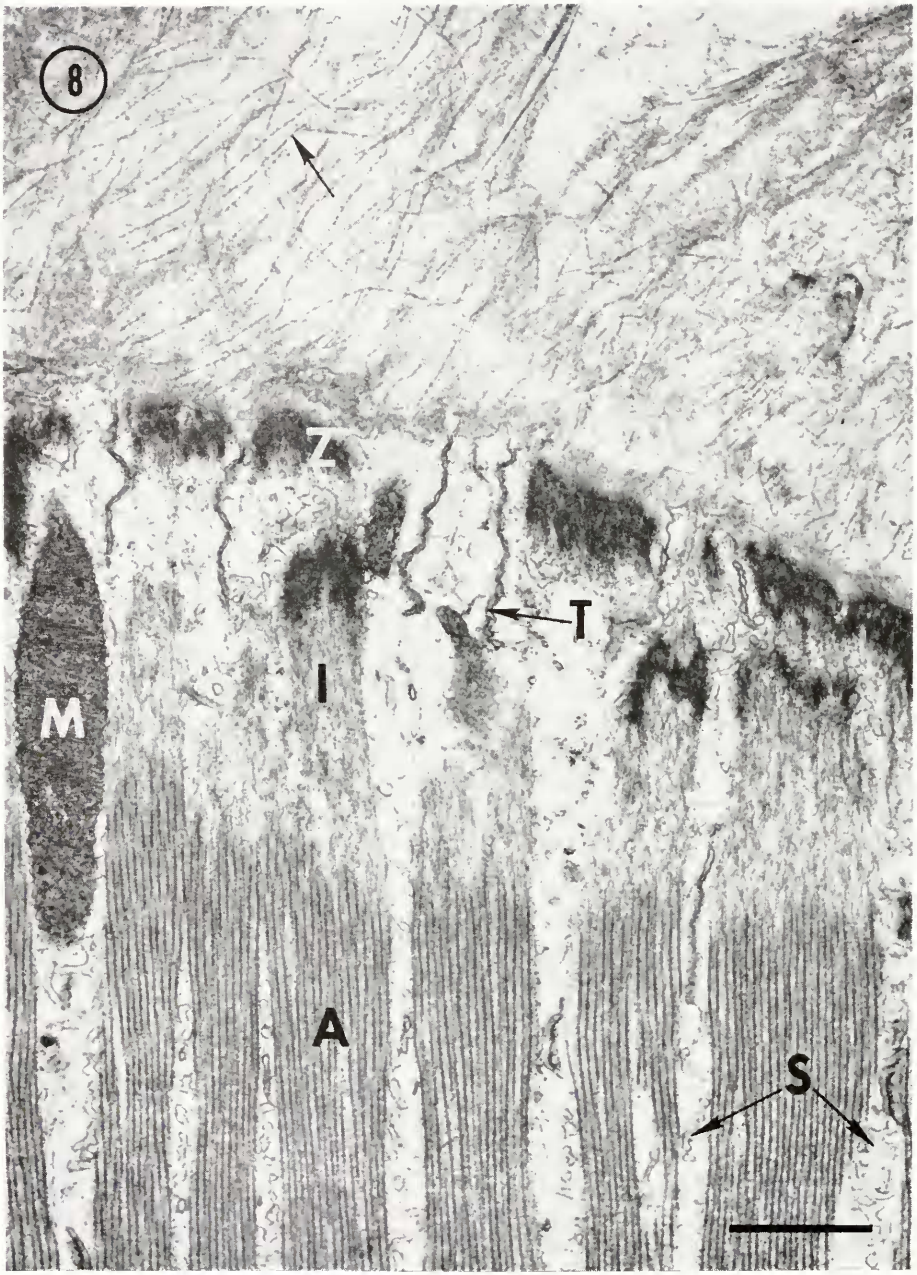

FIGURE 8. Portion of a muscle fiber at its origin showing the attachment of collagen fibrils (unlabeled arrow) to the muscle fiber. The fibrils extend into an amorphous, moderately dense material lining the external aspect of a thickened Z line (Z). The terminal Z line is two to three times the width of "normal" Z lines. This section is oblique, accounting for the

the muscle fiber Z lines. Here, the Z line is 0.2 to 0.4 μ in width, which is about two to three times the normal Z line width. Thin myofilaments join this thickened Z line as they do the Z lines in the muscle fiber interior. A moderately-dense layer of amorphous, basal lamina-like material lies on the extracellular surface of the thickened Z line. The collagen fibrils extend into this material and presumably anchor to it. Although the collagen fibrils are not strictly oriented, in general they lie perpendicular to the long axis of the thickened Z line.

Discussion

The attachment of propodite muscle fibers to skeletal elements in horseshoe crabs is achieved in two ways. At the origin, muscle fibers are attached directly to the cuticle by a network of collagen fibers that extend into the terminal Z lines. Attachment at the insertion is much more elaborate. Here, specialized epidermal cells, the tendinal cells, are interposed between the muscle fibers and apodemes. These elements are connected together in series through desmosomal junctions formed between thin myofilaments and microtubules on the one hand and on the other by hemidesmosomal junctions formed between microtubules and apodemes. In this manner, the contractile filaments are functionally connected to the apodemes, which in many cases are elongated to form tendons (Richards, 1951), by the vast numbers of oriented microtubules in the tendinal cells.

The structural details of the insertion of muscle onto skeletal elements in horseshoe crabs conform to those reported for most of the other arthropod groups. The presence of specialized epidermal cells intervening between muscle and cuticle has been reported for all of the arthropods studied (Lai-Fook, 1967; Caveney, 1969; Smith *et al.*, 1969; Kuo *et al.*, 1971; Koulish, 1973). Desmosomal and hemidesmosomal junctions between muscle and tendinal cell and tendinal cell and cuticle respectively also are typical of arthropods. However, some of the ultrastructural details of the attachment sites differ between the various arthropods.

In insects, in an acarine arachnid and larval stages of a malacostracan crustacean, the tendinal cells are attached to the cuticle by means of narrow elliptical sockets or cone-like depressions invaginated into the tendinal cell. These sockets are connected through hemidesmosomes to extracellular attachment fibers variously referred to as tonofibrillae, muscle attachment fibers and intracuticular fibers which extend into the cuticular layers where they end (Lai-Fook, 1967; Caveney, 1969; Kuo *et al.*, 1971; Talbot *et al.*, 1972). These extracellular fibers are not present in spiders (Smith *et al.*, 1969), scorpions (Mazurkiewicz and Bertke, 1972), barnacles (Koulish, 1973) or in the horseshoe crab material examined in the present study. Thus, the attachment zones in the latter organisms are somewhat simpler in organization.

Another notable difference found in the details of arthropod skeletal muscle insertions occurs in the acarine arachnids (mites). Here, the mechanical force exerted by muscles on the exoskeleton is not transmitted through microtubules, for these subcellular structures are absent from the intervening epidermal cells (Kuo

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apparent double Z line. Features typical of striated muscle are evident; namely, A band (A), I band (I), a mitochondrion (M), sarcoplasmic reticulum (S) and T-tubules (T). Calibration line represents 0.9 μ.

et al., 1971). Instead, only the specialized desmosomal junction is present which apparently is sufficient to provide the necessary intercellular linkage in these very small arthropods.

One particular feature of the desmosomal junctions involved in muscle attachments deserves special mention. The gap between the apposing plasma membranes at the desmosome is considerably wider in many of the arthropods examined than is usually encountered for this type of intercellular junction. The gap width typically is about 250 Å (Farquahar and Palade, 1963), whereas it may reach 700 Å in crustacean muscle attachments (Koulish, 1973), and gaps of 1,000 Å were commonly seen at horseshoe crab muscle insertions. The implications for the mechanical strength of desmosomes having such wide gaps remains to be assessed.

The elaborate extent of the cellular interdigitations at the attachment zones between muscle and tendinal cells and tendinal cells and apodemes results in a considerable increase in the total area available for intercellular mechanical linkage. Lai-Fook (1967) has estimated that the process of interdigitation increases the area of contact at the muscle-tendinal cell junction by a factor of from eight to ten and at the tendinal cell-apodeme junction by a factor of 40. No attempt was made to quantify this feature for horseshoe crab intercellular attachment zones, but a similar extent of interdigitation appears to be present in this organism as well.

The concentration of microtubules in the horseshoe crab tendinal cells is similar to that reported for these cells in an insect (Lai-Fook, 1967) and a spider (Smith *et al.*, 1969). A figure of $750/\mu^2$ was reported in the latter study which is comparable to the concentration seen in horseshoe crabs of $910/\mu^2$. In addition, there is no regular pattern of microtubule distribution in the tendinal cells of these arthropods.

The presence of gap and septate junctions between adjacent tendinal cells has not been reported previously for arthropods. Septate junctions are commonplace in invertebrate epithelial tissues and gap junctions have been found in a variety of tissues in several different animal phyla (*cf.* Hand and Gobel, 1972). A number of functions have been proposed for these specialized junctions. These include intercellular adhesion, intercellular chemical communication and intercellular electrical coupling. Since tendinal cells are primarily involved in the transmission of mechanical forces from muscle to exoskeleton, the tight and septate junctions in all probability provide a strong mechanical linkage between adjacent tendinal cells. Whether or not they provide sites for intercellular communication remains to be determined.

Comparisons between horseshoe crabs and other arthropods in the mode of muscle attachment to exoskeleton at muscle origins cannot be made, since most of the previous studies have not distinguished clearly between muscle origins and insertions. Therefore, it is not known if there are different modes of attachment between origins and insertions in other arthropods. A direct linkage of muscle to cuticle has been reported for a mite, but the precise details of the attachment were not given (Kuo *et al.*, 1971). The muscle responsible for venom expulsion in the black widow spider is attached directly to the fibrous sheath that surrounds the venom gland (Smith *et al.*, 1969). Here, the gland muscle is extensively invaginated by branches of the sheath, and the collagen fibrils in the sheath matrix are thought to provide a mechanical linkage between the myofibrils and the gland.

Summary

At their insertion, propodite flexor muscle fibers in horseshoe crabs are attached to apodemes via specialized epidermal cells, the tendinal cells, which contain tremendous numbers of microtubules. At the desmosomal muscle-tendinal cell junction, thin myofilaments attach to one side of the junction and tendinal cell microtubules to the other side. At the apical end of the tendinal cells, the microtubules extend into the dense material lining the hemidesmosomes formed by the tendinal cells and apodeme. In this manner, thin myofilaments are functionally connected to apodemes through intervening tendinal cell microtubules.

At the muscle origin, collagen fibrils embedded in a matrix connect terminal Z lines, which are two to three times the width of normal Z lines, to the surface cuticle. Tendinal cells and microtubules are not involved, and the attachment of muscle is directly to the exoskeleton.

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